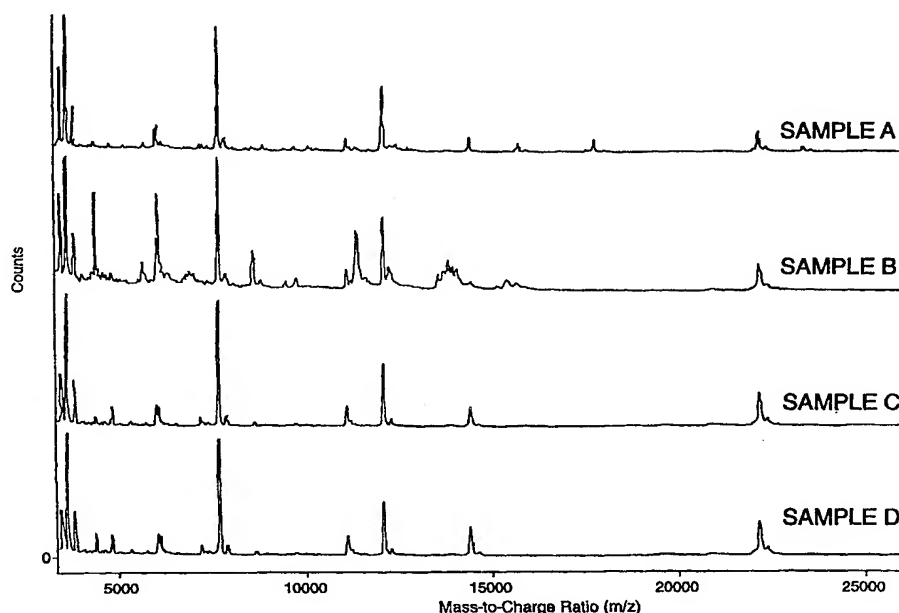




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(54) Title: METHOD FOR ASSESSING THE RELATIVE PURITY OF VIRAL GENE TRANSFER VECTOR STOCKS



**(57) Abstract**

The present method involves evaluating the purity of a viral gene transfer vector stock by providing an aliquot of the viral gene transfer vector stock and producing charged fragments from the aliquot. The fragments are detected by a detector that produces a sample signal corresponding to the mass-to-charge ratio of the detected fragments. This signal then is compared to a standard signal, whereby the purity of the viral gene transfer vector stock is evaluated.

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METHOD FOR ASSESSING THE RELATIVE PURITY OF VIRAL GENE  
TRANSFER VECTOR STOCKS

TECHNICAL FIELD OF THE INVENTION

5       The present invention relates to methods of  
assessing the relative purity of stocks of viral gene  
transfer vectors.

BACKGROUND OF THE INVENTION

10       Viral gene transfer vectors are widely used  
throughout molecular biology, especially in gene therapy.  
A major problem posed by the use of viral gene transfer  
vectors for gene therapy is the lack of consistent and  
effective methods to assess the purity of a stock of  
15   viral gene transfer vector. The lack of efficient means  
for determining purity of viral gene transfer vectors has  
resulted in the inability to measure the effectiveness or  
consistency of viral gene transfer vector production  
techniques. These problems are magnified when  
20   considering the safety concerns surrounding use of viral  
gene transfer vectors in human gene therapy.

Traditionally, attempts to measure the purity of an  
unmodified virus particle in a medium have relied on  
electrophoresis techniques, such as sodium dodecyl  
25   sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or  
ultraviolet (UV) spectroscopy measurements. Capillary  
gel electrophoresis also has been used to analyze viral  
components in comparison to known standards, as  
illustrated in U.S. Patent 5,723,031 (Durr et al.) In  
30   addition, ultraviolet spectroscopy techniques and  
ultraviolet resonance spectra have been used to provide  
structural analyses of several viruses.

Other techniques for assessing viral purity have  
centered on the use of chromatography techniques such as  
35   ion exchange chromatography, gel-filtration  
chromatography, and reverse phase high performance liquid  
chromatography (HPLC). For example, researchers have

utilized HPLC to purify structural proteins from Herpes Simplex Virus type-1. Similar techniques for purifying viral components have been developed using cation exchange chromatography as described in U.S. Patent 5,486,470 (Darke et al.), ion exchange chromatography as described in U.S. Patent 5,696,238 (Haigwood and Scandella), and electrical chromatography as described in U.S. Patent 5,707,850 (Cole and Gaithersburg).

Mass spectrometry, with the advantage of fast, accurate and detailed analysis as compared to several of the aforementioned techniques, has been used to study individual organic compounds. For example, Vastola et al., *Organic Mass Spectrometry*, 3, 101-104 (1969), disclosed the use of laser pyrolysis mass spectrometry to study the spectra generated by organic salts. Since that time, mass spectrometry has been widely used to evaluate features of larger organic molecules such as proteins. Recently these techniques have been used to study or validate the purity of individual viral proteins or groups of viral proteins such as the components and modifications of viral capsid proteins. For example, Fetzer et al., *Protein Expression and Purification*, 5, 432-441 (1994), teach the use of mass spectrometry in combination with SDS-PAGE to validate the purity of a herpes simplex virus thymidine kinase protein that was subjected to proteolytic cleavage and chromatography purification.

Other researchers have studied groups of viral proteins, for example, Davidson and Davidson, *Virology*, 206, 1035-1043 (1995), disclosed the identification of proteins of channel catfish virus by use of fractionation techniques in combination with laser desorption ionization mass spectrometry. Similarly, Pepinsky et al., *J. Virology*, 70, 3313-18 (1996), used electrospray mass spectrometry to obtain molecular masses for Gag proteins in the Rous sarcoma virus, to study protein

intermediates, and to determine that exopeptidase trimming occurred in some of the *Gag* proteins.

More recently, Bothner et al., *J. Biol. Chem.*, 273, 673-76 (1997), have disclosed the use of matrix assisted  
5 laser desorption/ionization mass spectrometry (MALDI or MALDI-MS), in combination with limited proteolysis, upon flock house virus particles to study viral capsid dynamics in that species. Also recently, mass spectrometry has been used as a tool for diagnosing  
10 specific DNA molecules in viruses, as described in U.S. Patent 5,605,798 (Koster).

Not until very recently has mass spectrometry been applied to entire virus particles. Tas et al., *Biomedical and Environmental Mass Spectrometry*, 18, 757-  
15 760 (1989), met with mixed results in attempting to characterize Vero cell cultures infected with herpes simplex virus and poliomyelitis virus by studying low-weight molecules (i.e., salt molecules, methyl groups, and sugars) that were detected by use of pyrolysis/direct  
20 chemical ionization (Py/DCI) mass spectrometry. While Tas et al. suggested that they could possibly identify cell lines infected with a particular virus, they failed to teach a method of obtaining relatively pure viral stocks, especially of a viral gene transfer vector  
25 capable of use in molecular biological applications, particularly gene therapy.

Despeyroux et al., *Rapid Communications in Mass Spectrom.*, 10, 937-41 (1996), were able to generate a spectrum for purified cricket paralysis virus (CrPV) by  
30 using electrospray ionization mass spectrometry and suggested that comparisons could be made to such spectra. However, Despeyroux et al.'s results are limited because success of their technique was attributed to the small size and simplicity of structural polypeptides of the  
35 cricket paralysis virus. Furthermore, similar to Tas et al., Despeyroux et al. failed to teach the combination of such techniques with a useful viral gene transfer vector.

Siuzdak et al., *Chemistry & Biology*, 3, 45-48 (1996), similarly used electrospray ionization (ESI) mass spectrometry, under special settings, to detect viruses. Using standard ESI techniques, Siuzdak et al. were not  
5 able to derive signals for large virus particles. Siuzdak et al. were capable of generating signals and performing mass selection on the charged particles from tobacco mosaic virus (TMV) and rice yellow motile virus (RYMV), by using the radio frequency (RF) mode of an ESI  
10 mass spectrometer in a manner that retained the ultrastructure of the virus particles. Thus, Siuzdak et al. taught that non-destructive ESI could actually purify virus particles. However, Siuzdak et al. only reported on small viruses (i.e., molecular weights of less than  
15 50,000,000 Daltons), and did not assess the relative purity of a virus that had not been modified by subjection to mass spectrometry.

Siuzdak, *J. Mass Spectrom.*, 33, 203-211 (1998), also disclosed that TOF mass spectrometry might be used to  
20 measure large ions such as a whole Tobacco Mosaic Virus. However, such work, if carried out, was not published, and nevertheless would not have provided a method of easily and efficiently assessing relative viral purity, especially in regard to large virus particles, such as  
25 with a stock of a useful viral gene transfer vector.

Moreover, many specific types of viral gene transfer vectors that have proven to be powerful tools in molecular biology often have molecular weights well in excess of those of TMV and RYMV. For example, adenoviral  
30 vectors, which are useful delivery vehicles, such as in gene therapy, have molecular weights often exceeding 150,000,000 Daltons, three times the weight of the viruses studied by Siuzdak et al.

Furthermore, the techniques taught by Tas et al.,  
35 Despeyroux et al., and Siuzdak (i.e., ESI and Py/DCI mass spectrometry) are difficult to perform, and require complicated analysis to evaluate their results.

Therefore, such techniques may not be a viable alternative for widespread use in the production of viral gene transfer vector stocks.

In view of the state of the art, there remains a  
5 need for methods to efficiently evaluate the purity of  
stocks of viral gene transfer vector. More particularly,  
there is a need to evaluate the relative purity of stocks  
of viral gene transfer vectors, suitable for carrying  
large amounts of genetic information for use in practical  
10 biological applications.

#### BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of  
evaluating the purity of a viral gene transfer vector  
15 stock. The present method of evaluating the purity of a  
viral gene transfer vector stock comprises providing an  
aliquot of a viral gene transfer vector stock and  
producing charged fragments from the aliquot. The  
fragments are detected by a detector that produces a  
20 sample signal corresponding to the mass-to-charge ratio  
of the detected fragments. This signal then is compared  
to a standard signal, whereby the purity of the viral  
gene transfer vector stock is evaluated.

#### 25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph of mass spectrometer traces of  
various viral gene transfer vector stocks plotted as  
mass-to-charge ratio ( $m/z$ ) (x-axis) versus relative  
counts (y-axis).

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#### DETAILED DESCRIPTION OF THE INVENTION

The present method involves evaluating the purity of  
a viral gene transfer vector stock by providing an  
aliquot of a viral gene transfer vector stock and  
35 producing charged fragments from the aliquot. The  
fragments are then detected by a detector, which produces  
a sample signal corresponding to the mass-to-charge ratio

of the detected fragments. A standard signal is provided, and the purity of the viral gene transfer vector stock is evaluated by comparing the sample signal to the standard signal.

5       The present invention is particularly useful in the context of purifying (i.e., substantially enriching a solution comprising) a viral gene transfer vector, for example, a recombinant adenoviral gene transfer vector.

10       The viral gene transfer vector can be any viral vector that contains at least one exogenous gene. A vector is any polynucleotide competent for introducing one or more exogenous genes into a cellular environment. One skilled in the art will recognize that the viral gene transfer vector (i.e., the viral vector as well as the  
15       exogenous gene) can be composed of single-stranded ribonucleic acid (RNA), double-stranded RNA, single-stranded deoxyribonucleic acid (DNA), or double-stranded DNA.

20       The viral vector can be any vector capable of containing recombinant nucleic acids that are (at least in part) of viral origin. A viral vector as indicated above can be any virus which contains DNA, for example, an adenoviral vector, or one that contains RNA, for example, a retrovirus. Examples of such vectors include,  
25       but are not limited to, adenoviral vectors (Bain et al., *Gene Therapy*, 1, S68 (1994)), adeno-associated viral vectors (Berns et al., *Annals of the New York Academy of Sciences*, 772, 95-104 (1995)), packaged amplicons (Ferreroff et al., *Proc. Nat. Acad. Sci. USA*, 89, 1636-40  
30       (1992)), and herpes simplex virus-based viral vectors (Fink et al., *Ann. Rev. Neurosci.*, 19, 265-87 (1996)).

35       Preferably, a viral vector is a vector derived from viral origin that contains nucleic acid sequences which encode for (a) the production of a capsid, a partial capsid, modified capsid, capsid protein, or modified capsid protein, any of which may or may not have all of the protein sub-units or sub-unit arrangement of the wild-



type viral capsid that corresponds to the origin of the viral vector or even identical sub-units to those expressed in the wild-type virus, (b) the functional portion of at least one terminal repeat, for example, the  
5 inverted terminal repeats found in adenoviruses or the long terminal repeats found in retroviruses, preferably two functional terminal repeats, including, but not limited to, long terminal repeats (LTRs) or inverted terminal repeats (ITRs) exhibited in viruses which assist  
10 in DNA replication, and (c) at least a partial viral packaging sequence, more preferably a sequence that enables the packaging of reproduced viral vector nucleic acids that are about 73% to 110% in size of the corresponding wild type viral genome.

15 More preferably, the sequences encoding for a capsid, partial capsid, modified capsid, capsid protein(s), or modified capsid protein(s) encode a protein arrangement that substantially shields the viral vector DNA from damage or degradation by enzymatic or other means.

20 The viral gene transfer vector can have any suitable molecular weight. Viral gene transfer vectors of substantial molecular weights (e.g., the combined mass of proteins, lipids, polysaccharides, and nucleic acids, including the exogenous gene, that comprise the viral gene  
25 transfer vector) can be evaluated within the context of the present method. For example, the viral gene transfer vector can possess a molecular weight of at least 1,000,000 Daltons, 3,000,000 Daltons, 20,000,000 Daltons, 50,000,000 Daltons, 100,000,000 Daltons, 150,000,000  
30 Daltons, 500,000,000 Daltons, 1,000,000,000 Daltons, or even 1,500,000,000 Daltons.

Similarly, the viral gene transfer vector can have any suitable genome size comprised of nucleic acid sequences or base pair sequences native to the viral  
35 vector and those that comprise the exogenous gene or genes. Viral gene transfer vectors of large genomes can be evaluated within the context of the present method.

For example, the viral gene transfer vector can have a total genome of at least 2,000 nucleotides or nucleic acid base pairs, 4,000 nucleotides or nucleic acid base pairs, 7,000 nucleotides or nucleic acid base pairs, 20,000  
5 nucleotides or nucleic acid base pairs, 30,000 nucleotides or nucleic acid base pairs, or even 100,000 nucleotides or nucleic acid base pairs.

The exogenous gene in the context of the present invention includes any exogenous nucleic acid that  
10 encodes a desired polypeptide or ribonucleic acid, is operatively linked to a suitable promoter, and can be expressed to produce the desired polypeptide or ribonucleic acid. For example, the exogenous gene can comprise a nucleotide sequence that can be expressed to  
15 produce a polypeptide, a ribozyme, or antisense RNA molecule. The exogenous nucleic acid is not native to the viral vector, although the promoter operatively linked thereto may or may not be native to the viral vector. Thus, the viral gene transfer vector is not a  
20 wild-type virus. The polypeptides or ribonucleic acid encoded by the exogenous nucleic acid can be any suitable polypeptide or ribonucleic acid, such as a reporter polypeptide or ribonucleic acid (i.e., the exogenous gene can be a reporter gene), and preferably a therapeutic  
25 polypeptide or ribonucleic acid (i.e., the exogenous gene can be a therapeutic gene).

The present invention involves the evaluation of a stock of the viral gene transfer vector. The viral gene transfer vector stock can be any composition comprising a  
30 viral gene transfer vector and a suitable carrier therefor. The choice of carrier will be determined in part by the particular end use of the viral gene transfer vector stock. The carrier preferably is a pharmaceutically acceptable carrier. A pharmaceutically  
35 acceptable carrier typically will be a substance useful in the administration of the viral gene transfer vector to an animal, such as a human, for therapeutic treatment.

Pharmaceutically acceptable carriers are known to those skilled in the art and are readily available.

A viral gene transfer vector stock can be produced by transfecting a viral gene transfer vector into a cell  
5 (i.e., a production cell) that is permissive for the replication of the viral gene transfer vector to produce a transfected cell. The permissive cell is ordinarily maintained in a medium that promotes cell growth, examples of which are widely known in the art. The  
10 transfected cell is maintained in the medium so as to cause the viral gene transfer vector to replicate. The viral gene transfer vector will ordinarily be separated from the cell and from the medium to produce a stock of a viral gene transfer vector.

15 Preferably, the viral gene transfer vector stock is obtained by infecting eukaryotic cells with a viral gene transfer vector of interest, maintaining the cells for a period of time sufficient to amplify the number of viral gene transfer vector particles, gathering the infected  
20 cells together, and lysing (i.e., breaking open) the cells in a buffered solution. The viral gene transfer vector stock can be subsequently treated or modified in any suitable manner. For example, purification or separation techniques can be performed on the viral gene  
25 transfer vector stock to remove the viral gene transfer vector from the cells and the medium, thereby yielding a more concentrated stock of the viral gene transfer vector. Such purification and separation techniques are well known in the art. Such techniques can include, but  
30 are not limited to, chromatography, electrophoresis, enzymatic or chemical treatment, exposure to heat or radiation, centrifugation, and separation by a differential gradient, for example, a cesium chloride gradient.

35 The viral gene transfer vector stock can have any suitable level of purification or concentration. For example, the present method can be practiced on

unmodified stocks of viral gene transfer vectors which can include undesired substances or molecules, as well as stocks that are considered substantially pure. More specifically, such undesired substances or molecules can  
5 include, but are not limited to, the cell, other components of the stock, for example, an undesired buffering agent, and inorganic and organic molecules or polymers, such as biomolecules from the viral gene transfer vector or other living organisms or virus  
10 particles, such as nucleic acids, lipids, polysaccharides, and proteins.

The production cell can be any cell permissive of the reproduction of the viral gene transfer vector. The production cell preferably is a eukaryotic cell, more  
15 preferably a mammalian cell, and most preferably a human cell. The production cell can be a primary or secondary cell or can originate from a cell line, for example, an immortalized cell line, or a cell line that expresses an immortalized phenotype or characteristics. Such  
20 characteristics can be used as a marker, for example, by selecting cells that exhibit such characteristics. Alternatively, or in combination, the production cell can be a cell that expresses a marker gene that allows for either positive or negative selection based upon the  
25 expression of the marker gene.

The production cell can have any morphological characteristics that allow reproduction of the viral gene transfer vector in that particular cell. For example, the production cell can be epithelial or epithelial-like  
30 in morphology, such as cells of, or derived from, human kidney, lung, or neural cells. The production cell also can be an embryonic cell, preferably a human embryonic cell, for example, a human embryonic kidney cell, human embryonic lung cell, or human embryonic retinoblast.

35 The production cell can be a cell that has been modified to express nucleic acids that partially *trans*-complement the viral gene transfer vector, especially

when the viral gene transfer vector otherwise is replication incompetent. For example, the production cell can be a cell that *trans*-complements a replication incompetent viral gene transfer vector so as to prevent undesired recombination between the genome of the production cell and the viral gene transfer vector, e.g., to prevent a homologous recombination event between the genome of the production cell and the viral gene transfer vector, while allowing the reproduction of the viral vector in the production cell.

An aliquot can be taken from the viral gene transfer vector stock by any suitable technique. Charged fragments (e.g., ionized fragments) are produced from the aliquot by any suitable technique. If desired, the aliquot can be appropriately treated prior to production of the charged fragments therefrom. For example, the aliquot can be treated to reduce the salt level therein (e.g., from a physiological level of 150 mM salt to 50 mM salt or less) to reduce background noise in subsequently detecting the fragments, even if the viral gene transfer vector stock itself will not be so treated, so long as the subsequent comparison of the sample and standard signals is not adversely affected. The present method can be performed using techniques that allow fragmentation without the use of chemical ionization methods. For example, the present technique does not require the use of pyrolysis direct/chemical ionization (PY/DCI) mass spectrometry, or other forms of chemical ionization mass spectrometry, to produce suitable charged fragments.

While the charged fragments can be produced by any suitable technique, the charged fragments typically will be produced by contacting the aliquot with radiation, electrons, or protons, preferably protons. Any suitable form of radiation, electrons, or protons can be used in the present method to produce charged fragments from the aliquot. Radiation as used in the context of the present

invention refers to the emission of energy in the form of electromagnetic waves, acoustic waves, or particles. Examples of radiation suitable in the context of the present invention include, but are not limited to, radio waves, microwaves, visible light, ultraviolet (UV) light, far UV and infrared rays, x-rays, gamma-rays, infrasonic waves, sonic waves, ultrasonic waves, and  $\alpha$ - and  $\beta$ -rays of radioactivity. The electrons can be in any suitable form. For example, the electrons can be in the form of electron beams or individual electrons. Similarly, the protons can be in any suitable form.

The radiation, electrons, and protons can be from any suitable source. For example, radiation can be emitted from a natural source (e.g., radioactive cobalt), an x-ray device, or a laser.

The radiation, electrons, and protons can have any suitable characteristics. In particular, the radiation can have any suitable wavelength. For example, the method can incorporate a radiation pulse that has a wavelength in the infrared spectrum or the ultraviolet spectrum. Similarly, any pulse width suitable to produce fragments can be utilized in the present method. For example, the pulse generated by a laser can have a width of up to about 100, 200, or even 5,000 nanoseconds. Moreover, variable pulse widths and multiple repeated pulses can be used to produce fragments.

The produced fragments can be in any state or quantity suitable for fragment detection. For example, the aliquot can be vaporized at the time the charged fragments are produced from the aliquot, such that the fragments enter into a gaseous or vapor state, have greater mobility, and are subject to easier detection. The fragments can have any suitable velocity at the time of production that allows for detection by the detector. For example, fragments can be accelerated, after they are produced, to a desired velocity before the fragments are detected. The fragments can pass through a field-free

region, wherein the velocity of the fragments within the field-free region is proportional to the mass-to-charge ratio of the fragments. One example of such a field-free region is an electric field, particularly an electric  
5 field wherein lighter fragments have a higher velocity than heavier fragments. While acceleration of fragments is not required, if the fragments are accelerated, any level of acceleration sufficient for detection of the fragments is suitable. For example, the fragments can be  
10 accelerated to a fixed kinetic energy by contacting the fragments with an electric potential.

A viral vector typically will comprise proteins that are associated (or bound) to each other, as well as nucleic acids, which together form the ultrastructure of  
15 the viral vector. As a result of contact with the protons, the proteins can become disassociated from one another (but otherwise remain substantially intact) and/or the proteins can be further fragmented to yield portions of the proteins. A significant amount of  
20 information can be obtained about the viral gene transfer vector stock by fragmenting the individual proteins into portions of the proteins; however, the analysis of such information can be complex and time-consuming. It has been found that the mere disassociation of the viral  
25 vector proteins, e.g., merely causing the viral gene transfer vector to lose its ultrastructure through the disassociation of, for example, viral capsid or modified capsid proteins, such that the produced fragments of the viral gene transfer vector comprise, preferably consist  
30 essentially of, and more preferably consist of, substantially or entirely intact viral vector proteins typically is sufficient for purposes of present invention and provides information that can be easily and quickly analyzed.

35 The detection of the produced fragments can be accomplished by any suitable technique. The detection step can include, for example, measuring the time-of-

flight for the detected fragments, wherein the time-of-flight is the approximate time required for a fragment to travel a distance across a field-free region. A signal corresponding to the time-of-flight then can be

5 generated.

While the fragmentation and detection steps of the present method can be performed in any suitable manner, they are preferably performed by the use of an analytical device, most preferably by the use of an ion source and a mass analyzer, such as are present in mass spectrometers. Suitable ion sources include electron impact, fast ion or atom bombardment, field desorption, laser desorption, plasma desorption (PD), thermospray (TSP), electrospray ionization (ESI) (including, but not limited to, nanoelectrospray ionization and/or capillary electrospray), inductively coupled plasma (ICP), and atmospheric pressure ionization (API) (including, but not limited to, atmospheric pressure chemical ionization (APCI)). Preferred ion sources include laser desorption, thermospray (TSP), electrospray ionization (ESI), and atmospheric pressure ionization (API). Suitable mass analyzers include quadrupolar analyzers, quadrupole ion trap or quistor analyzers, time-of-flight (TOF) analyzers, magnetic and electromagnetic analyzers, and ion cyclotron resonance analyzers, and Fourier transform mass analyzers. The quadrupolar analyzers are preferred, especially in connection with electrospray ionization (e.g., a triple quadrupole analyzer). The time-of-flight analyzers are more preferable in the context of the present invention, with the most preferred devices for producing and detecting fragments in the context of the present invention being Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometers. Other mass spectrometers that can be used in the context of the present invention include an electrospray ionization mass spectrometer, a plasma desorption spectrometer, a thermospray ionization spectrometer, and

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a laser desorption mass spectrometer. A spectrometer that combines these techniques also can be suitable under appropriate conditions. For example, recent advances in the field have allowed the production of spectrometers which combine electrospray ionization with time-of-flight (or MALDI-TOF) analysis. One skilled in the art will recognize that the present invention can readily be practiced with such a spectrometer.

One skilled in the art will further recognize that improved resolution and faster analysis can sometimes be obtained using tandem mass spectrometry. Additionally, by using tandem mass spectrometry (e.g., by using a spectrometer possessing two detectors and a reflectron), identification of monoisotopic masses versus average masses is possible.

The fragments can have any suitable size for detection and sample signal production and analysis. Relatively large fragments can be detected upon initial fragmentation, without further fragmentation. For example, the produced and detected fragments can have molecular weights of up to 5,000 Daltons (i.e., atomic mass units or amu), 30,000 Daltons, 150,000 Daltons, or even 300,000 Daltons or more. Thus, multiple fragmentation events can be avoided prior to fragment detection or sample signal production and analysis.

Similarly, the fragments can have any suitable mass-to-charge ratio for detection and sample signal production and analysis. Fragments with relatively large mass-to-charge ratios can be detected, and sample signals produced and analyzed therefor. For example, the produced and detected fragments can have mass-to-charge ratios ( $m/z$ ) of at least 100, 200, 400, 500, 1,000, 2,500, 5,000, 10,000, 20,000, 25,000, or even 300,000 or more.

The sample signal generated upon detection of the fragments can be any type of sample signal that allows for comparison to a standard signal. The sample signal

can be generated in any suitable manner. Similarly, the standard signal can be any signal and in any form that allows for useful comparison with the sample signal to evaluate the relative purity of the viral gene transfer vector stock. The standard signal can be a single standard signal or a series or group of standard signals indicative of a range of viral gene transfer vector stock purity levels. The sample and standard signals can be associated with any suitable single mass-to-charge ratio and/or any suitable range(s) of mass-to-charge ratios. The sample signal and standard signal can be presented in similar or different (though preferably similar) formats, measurements, or units as long as a useful comparison can be performed. For example, a suitable standard signal can be a signal that is produced from techniques similar to those that are used to generate the sample signal. More specifically, the standard signal can be a signal that is generated from a standard source, e.g., a viral gene transfer vector stock of a known and/or desired relative purity.

The purity of the viral gene transfer vector stock is evaluated by comparing the sample signal to the standard signal in any suitable manner. For example, sample signal and standard signal traces of mass-to-charge ratio versus counts can be compared with respect to the presence, absence, and/or nature of one, some, or all of the peaks of the traces, which differences in the traces reflects the relative purity of the viral gene transfer vector stock.

The present method also can detect and characterize (e.g., identify) one or more contaminants in the viral gene transfer vector stock. For example, the present method can detect and characterize contaminants in the viral gene transfer stock by comparison of the standard and sample signals. The contaminant can have any suitable molecular weight, such as a molecular weight of at least 1,000 Daltons, 3,000 Daltons, 5,000 Daltons,

10,000 Daltons, 30,000 Daltons, 100,000 Daltons, 200,000 Daltons, 500,000 Daltons, or even 1,000,000 Daltons or more. Furthermore, the contaminant can have any suitable mass-to-charge ratio ( $m/z$ ), such as a mass-to-charge  
5 ratio of at least 100, 200, 400, 500, 1,000, 2,500, 5,000, 10,000, 20,000, or even 25,000 or more.

The contaminants can be any suitable type of undesired molecule or composition that can be detected, including, but not limited to, components of the viral  
10 gene transfer vector stock. For example, the contaminant can be any organic or inorganic molecule, compound, or polymer, or a portion thereof. More specifically, the contaminant can be a protein, nucleic acid, polysaccharide, or a lipid.

15 Upon detection of one or more contaminants, the contaminant(s) can be characterized by any suitable technique. For example, the contaminant can be characterized by comparison with the standard signal, or by other techniques such as, but not limited to,  
20 subjecting the contaminant to chromatography, differential gradient analysis (e.g., a cesium chloride gradient column or sucrose gradient), mass spectrometry, electrophoresis, centrifugation, or any combination of the aforementioned or other techniques.

25 Moreover, the contaminant(s) can be removed from the aliquot or viral gene transfer vector stock. This can be accomplished by any suitable technique, such as by any of the techniques referenced above.

In one aspect of the invention, the characterization  
30 of the contaminant (or contaminants) identified can be achieved through subjecting one or more fragments corresponding to the contaminant to further fragmentation and analysis. In this aspect, charged fragments are produced from a fragment of the contaminant (i.e.,  
35 secondary charged contaminant fragments). The secondary charged contaminant fragment then can be detected by a detector that produces a signal corresponding to the

mass-to-charge ratio of the detected secondary contaminant fragment. This signal (e.g., a spectrum) corresponding to the secondary charged contaminant fragment allows rapid identification of the contaminant.

5 Any suitable technique or combination of techniques can be used to produce charged fragments from the contaminant fragment, detect the charged secondary contaminant fragment, and generate a signal corresponding to the secondary charged contaminant fragment. For  
10 example, the contaminant fragment can be contacted with protons, electrons, or radiation to produce the secondary charged contaminant fragment or, preferably, be subjected to collision induced disassociation (CID). Detection of the secondary charged contaminant fragment is preferably  
15 performed by the use of an analytical device, most preferably by the use of an ion source and a mass analyzer, such as are present in mass spectrometers. More preferably, a MALDI-TOF spectrometer with precursor selector (or "ion gate") capabilities, a quadrupole  
20 electrospray spectrometer, or any spectrometer capable of focussing on one or more signals that correspond to the contaminant (or contaminants) in the stock is used to produce, detect, and analyze the secondary charged contaminant fragment. A MALDI-TOF spectrometer with  
25 precursor selector capabilities is particularly preferred because it is capable of accurately detecting (and focusing) on a signal corresponding to the contaminant, isolating the contaminant fragment, and rapidly performing further analysis on the isolated contaminant  
30 fragment.

An example of this aspect of the invention is the identification of the amino acid composition of a polypeptide contaminant by the above technique. After identifying a signal (or signals) corresponding to the  
35 polypeptide contaminant, the polypeptide can be further fragmented (e.g., by contact with radiation). By controlling fragmentation, fragments corresponding to

individual constituent amino acids can be produced. Signals corresponding to the amino acids then can be generated, allowing rapid determination of the amino acid composition of the polypeptide.

5           A similar technique can be used as a method to provide more detailed information regarding the purity of the viral gene transfer vector within the stock. For example, one or more signals within the sample signal that correspond to a fragment of the viral gene transfer  
10 vector (i.e., a standard fragment) can be identified. The standard fragment then can be further fragmented (e.g., by subjecting the standard fragment to radiation) to produce at least one secondary charged standard fragment from the standard fragment. The secondary  
15 charged standard fragment can be detected by a detector which produces a signal corresponding to the mass-to-charge ratio of the detected secondary standard fragments. This signal can allow identification of the components of the standard fragment (e.g., the amino acid  
20 composition of a viral protein of interest), similar to the technique described above for identifying specific contaminants. This technique can be particularly useful in ensuring the presence (or absence) of certain gene products in the viral gene transfer vector stock. While  
25 any suitable combination of techniques can be used to produce charged fragments from the standard fragment, detect the secondary charged standard fragment, and generate a signal corresponding to the secondary charged standard fragment, the preferred techniques described  
30 above relating to the contaminant fragment also are applicable to this aspect of the invention.

          In some instances it can be desirable to combine fragmentation (of the contaminant fragment or standard fragment) with supplemental fragmentation techniques.  
35 Suitable supplemental fragmentation techniques include collision-induced disassociation (CID), post source decay (PSD), and combinations thereof.

Accuracy can sometimes be improved by isolating the contaminant fragment or standard fragment, or a signal corresponding thereto, prior to further fragmentation. For example, the present inventive method can include a step of substantially isolating the contaminant fragment before producing a secondary charged contaminant fragment from the contaminant fragment. Alternatively a signal or signals known to correspond to the contaminant fragment can be isolated (i.e., from other signals in the sample signal) and focused on before producing a secondary charged contaminant fragment from the contaminant fragment. Similar techniques can be applied to the standard fragment. Any suitable method can be used to isolate the contaminant fragment or standard fragment prior to producing the secondary charged fragment. Similarly, any suitable method can be used to isolate a signal corresponding to the contaminant fragment or standard fragment. Preferably, isolating a signal corresponding to the contaminant fragment or standard fragment is done by using a MALDI-TOF spectrometer with ion trap capabilities, a quadrupole electrospray spectrometer, or any spectrometer capable of focussing on one or more signals corresponding to the contaminant fragment or standard fragment.

Any of the individual steps of the present method can be repeated two or more times. For example, the production of fragments and the detection of the fragments can be repeated two or more times to provide a satisfactory sample signal(s). Similarly, multiple viral gene transfer stocks can be provided, an aliquot can be obtained from each such viral gene transfer vector stock, and a sample signal can be produced for each such aliquot, thereby enabling the selection of stocks that have a desired purity based on comparison of the sample signals to the standard signal.

The aliquots can be taken from stocks that have been subjected to sequential cycles of separating the viral

gene transfer vector from the production cell and medium. After analysis of the sample signals by comparison with a standard signal, as described above, stocks that have a desired purity can be selected based on the comparison of the sample signals to the standard signal. In this fashion, for example, the viral gene transfer vector stock purification process can be evaluated or "benchmarked."

10

## EXAMPLE

This example further illustrates the present invention but should not be construed to limit the present invention in any way. Although this example is recited using particular aspects of the invention, for example, particular viral gene transfer vector stocks, as well as fragmentation and detection techniques, the skilled artisan will appreciate that the present inventive method can be applied to a wide range of viral gene transfer vector stocks, using a wide variety of techniques as described above.

20

Specifically, four different adenoviral vector stocks were provided. The adenoviral vector in each stock was an adenovirus of serotype 5 (i.e., Ad5) and contained an exogenous gene. The carrier in each stock was the same and was a physiologically compatible aqueous solution. The adenoviral vector stocks differed as to the manner in which each was purified from the production cells and growth media. The first two adenoviral vector stocks, Samples A and B, were prepared using standard chromatography techniques. The third adenoviral vector stock, Sample C, was prepared using three successive cesium chloride gradients and was considered to represent an adenoviral vector stock of a desirably high degree of purity. The fourth adenoviral vector stock, Sample D, was prepared using a nonconventional anion exchange chromatography matrix technique.

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35

An aliquot was obtained from each of the four adenoviral vector stocks. Each aliquot was treated to reduce its salt concentration, thereby reducing the noise in the subsequent sample signal. Each aliquot was  
5 subjected to MALDI-TOF mass spectrometry so as to produce and detect fragments from the aliquot. The mass spectrometry settings were such that the produced fragments comprised substantially intact proteins disassociated from the adenoviral vector, rather than  
10 portions of those proteins. The sample signal produced from the detection of the produced fragments resulted in a mass spectrometer trace for each of the aliquots of the four adenoviral vector stocks. These mass spectrometer traces are set forth in Figure 1 in a graph of mass-to-charge ratio ( $m/z$ ) (x-axis) versus relative counts (y-axis). Since the third adenoviral vector stock, Sample C, reflected the desired level of purity of the adenoviral vector, the Sample C mass spectrometer trace represented the "standard signal" in accordance with the  
20 present invention for purposes of considering the purity of the other adenoviral vector stocks, Samples A, B, and D. The Sample A, B, and D mass spectrometer traces were treated as the "sample signals" in accordance with the present invention.

25 As is apparent from the mass spectrometer traces of Figure 1, the fourth adenoviral vector stock, Sample D, is approximately as pure as the "control" adenoviral vector stock, Sample C. The other adenoviral vector stocks, Samples A and B, are not as pure as the "control" adenoviral vector stock, Sample C, with Sample B being  
30 less pure than Sample A. This is readily apparent from a comparison of the traces, e.g., the presence, absence, and nature of the peaks of the traces.

In the Sample C "standard signal," the peaks at, for  
35 example, approximately 11000, 12000, and 14500 mass-to-charge ratios ( $m/z$ ) are quite sharp. This is also true with respect to the Sample D "sample signal" as compared



to the Sample C "standard signal." Moreover, there are no significant additional or missing peaks in the Sample D "sample signal" as compared to the Sample C "standard signal." This similarity of the Sample D "sample signal" and the Sample C "standard signal" reflects a similar degree of purity of the adenoviral vector stocks.

In contrast, the peaks at approximately 11000, 12000, and 14500 mass-to-charge ratios ( $m/z$ ) are much broader in the Sample A and Sample B "sample signals" as compared to the Sample C "standard signal." Moreover, there are significant additional peaks in the Sample A "sample signal" at, for example, approximately 17500 and 23000 mass-to-charge ratios ( $m/z$ ), and in the Sample B "sample signal" at, for example, approximately 11500 and 13500 mass-to-charge ratios ( $m/z$ ), as compared to the Sample C "standard signal." In addition, there are significant missing peaks in the Sample A and Sample B "sample signals" at, for example, 4500 mass-to-charge ratio ( $m/z$ ), as compared to the Sample C "standard signal." This lack of similarity of the Sample A "sample signal" and the Sample C "standard signal," and the even greater lack of similarity of the Sample B "sample signal" and the Sample C "standard signal," reflect the presence of a higher level of contaminants in those adenoviral vectors stocks (i.e., adenoviral vector stocks of lower purity), more so with respect to Sample B than Sample A, as compared to the "control" adenoviral vector stock Sample C.

Thus, it is readily apparent from the results of this example that the present invention is quite useful in assessing the relative purity of viral gene transfer vector stocks.

All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that other variations of the invention can be used and that it is intended that  
5 the invention can be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of evaluating the purity of a viral gene transfer vector stock, the method comprising:

- 5 (a) providing an aliquot of a viral gene transfer vector stock;
- (b) producing charged fragments from the aliquot;
- (c) detecting the fragments by a detector which produces a sample signal corresponding to the mass-to-charge ratio of the detected fragments;
- 10 (d) providing a standard signal; and
- (e) evaluating the purity of the viral gene transfer vector stock by comparing the sample signal to the standard signal.

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2. The method of claim 1, wherein providing an aliquot of a stock of a viral gene transfer vector in step (a) of the method comprises:

- (a1) transfecting a viral gene transfer vector into
- 20 a cell that is permissive for the replication of the viral gene transfer vector to produce a transfected cell, wherein the cell is in a medium;
- (a2) maintaining the transfected cell in the medium so as to cause the viral gene transfer vector to
- 25 replicate;
- (a3) separating the viral gene transfer vector from the cell and from the medium to produce a stock of a viral gene transfer vector; and
- (a4) obtaining an aliquot from the stock.

30

3. The method of claim 2, wherein the method further comprises:

- (a5) repeating steps (a3) and (a4) a desired number of times to obtain a series of viral gene transfer vector
- 35 stocks and aliquots thereof;

(f) performing steps (a)-(e) on each aliquot obtained in step (a5) to produce a set of sample signals; and

5 (g) selecting stocks that have a desired purity based on the comparison of the sample signals to the standard signal.

10 4 The method of any of claims 1-3, wherein steps (b) and (c) are repeated two or more times.

5. The method of claim 4, wherein steps (b) and (c) are performed by tandem mass spectroscopy.

15 6. The method of any of claims 1-4, wherein steps (b) and (c) are performed by a mass spectrometer selected from the group consisting of an electrospray ionization mass spectrophotometer, a plasma desorption spectrophotometer, a thermospray ionization spectrophotometer, and a laser desorption mass  
20 spectrophotometer.

7. The method of any of claims 1-4, wherein steps (b)-(d) are performed by Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) spectrophotometry.  
25

8. The method of any of claims 1-4, wherein steps (b) and (c) are performed by a spectrometer capable of electrospray ionization and time-of-flight analysis.  
30

9. The method of any of claims 1-8, wherein the viral gene transfer vector is an adenoviral vector.

35 10. The method of any of claims 1-9, wherein the viral gene transfer vector comprises proteins associated with each other, and the fragments of the viral gene

transfer vector comprise substantially intact proteins disassociated from the viral gene transfer vector.

11. The method of any of claims 1-10, wherein the  
5 fragments of the viral gene transfer vector consist essentially of substantially intact proteins disassociated from the viral gene transfer vector.

12. The method of any of claims 1-11, wherein the  
10 comparison of the sample signal to the standard signal in step (e) further comprises the step of detecting the presence of a contaminant in the viral gene transfer vector stock.

13. The method of claim 12, wherein the method  
15 further comprises:

(h) characterizing the contaminant.

14. The method of claim 13, wherein the  
20 characterization of the contaminant in step (h) of the method comprises:

(h1) identifying at least one signal in the sample  
signal corresponding to a contaminant fragment;

(h2) producing at least one secondary charged  
25 contaminant fragment from the contaminant fragment;

(h3) detecting the secondary charged contaminant  
fragments by a detector which produces a signal  
corresponding to the mass-to-charge ratio of the  
detected secondary charged contaminant fragments.

30

15. The method of any of claims 12-14, wherein the  
method further comprises the step of removing the  
contaminant from the viral gene transfer vector stock.

16. The method of any of claims 1-15, wherein the  
35 viral gene transfer vector has a molecular weight at  
least 100,000,000 Daltons.

17. The method of any of claims 1-16, wherein the viral gene transfer vector is a vector that contains more than 4,000 nucleotides or nucleic acid base pairs.

5

18. The method of any of claims 1-17, wherein the sample signal comprises a signal produced from a fragment having a molecular weight of up to 300,000 Daltons when the fragment is detected.

10

19. The method of any of claims 1-18, wherein the sample signal comprises a signal produced from a fragment having a mass-to-charge ratio of at least 200 when the fragment is detected.

15

20. The method of any of claims 1-19, wherein the standard signal is provided by a viral gene transfer vector stock of known purity.

20

21. The method of any of claims 1-20, wherein the standard signal is provided in the same manner as the sample signal.

22. The method of any of claims 1-21, wherein the fragments pass through a field-free region, wherein the velocity of the fragments within the field-free region is proportional to the mass-to-charge ratio of the fragments.

23. The method of claim 22, wherein the detection of the fragments in step (c) further comprises:

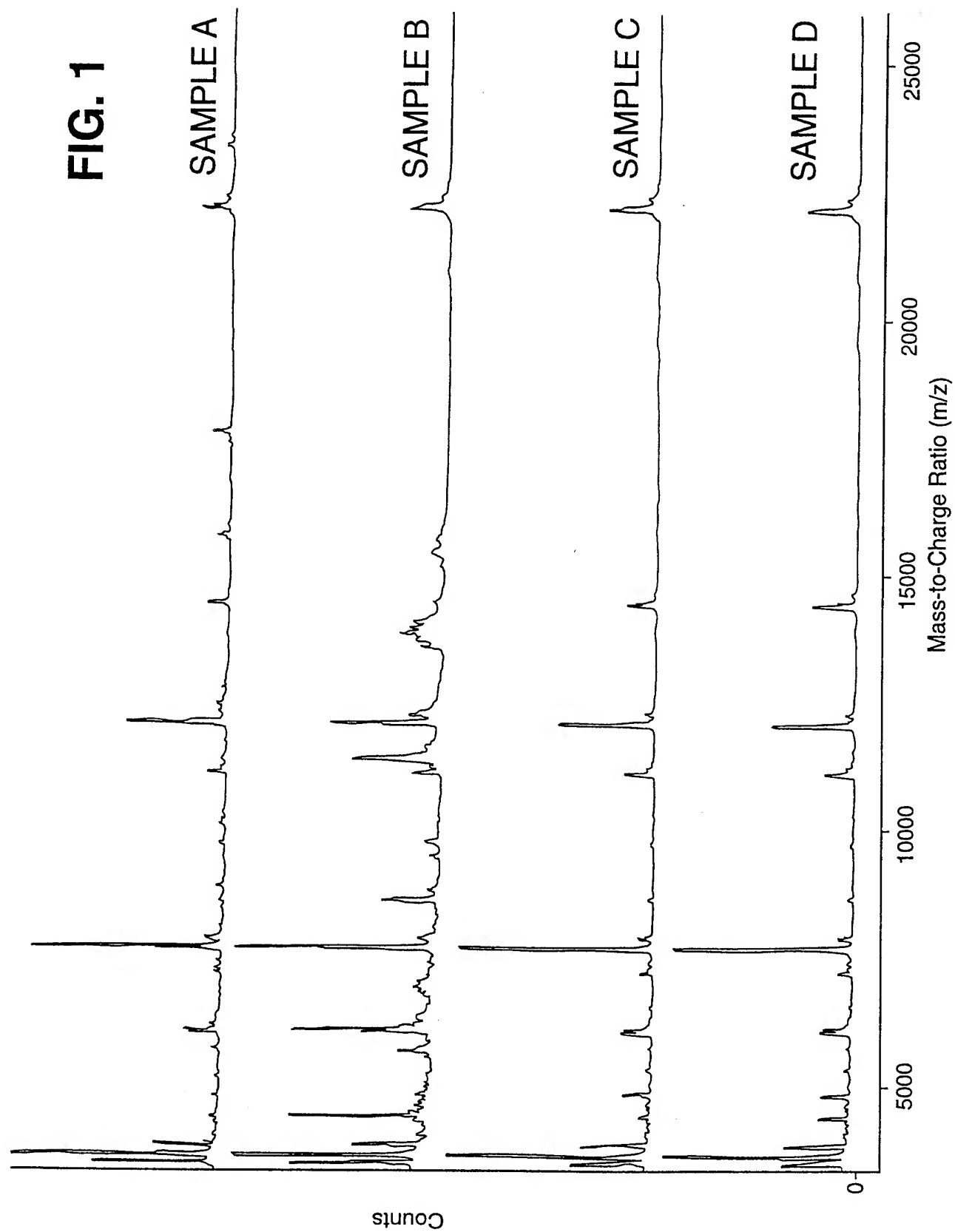
(c1) measuring the time-of-flight for the detected fragments, wherein the time-of-flight is the approximate time required for a fragment to travel a distance across the field-free region; and

(c2) producing a sample signal corresponding to the time-of-flight.

24. The method of any of claims 1-23 wherein step (e) of the method further comprises:

- 5 (e1) identifying at least one signal in the sample signal corresponding to a standard fragment;
- (e2) producing at least one secondary charged standard fragment from the standard fragment; and
- 10 (e3) detecting the secondary charged standard fragments by a detector which produces a signal corresponding to the mass-to-charge ratio of the detected secondary charged standard fragments.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/18131

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SIUZDAK: "PROBING VIRUSES WITH MASS SPECTROMETRY"  J.MASS.SPECTROM.,  vol. 33, March 1998 (1998-03), pages  203-211, XP000863342  cited in the application  the whole document  &amp; DATABASE HCA 'Online!  RAPID COMMUN. MASS SPECTROM., 1995  FUERSTENAU ET AL.: "MOLECULAR WEIGHT  DETERMINATION OF MEGADALTON DNA  ELECTROSPRAY IONS USING CHARGE DETECTION  TIME-OF-FLIGHT MASS SPECTROMETRY"  abstract</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-24



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

In. itional Application No

PCT/US 99/18131

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>SIUZDAK ET AL.: "MASS SPECTROMETRY AND VIRAL ANALYSIS" CHEMISTRY&amp;BIOLOGY, vol. 3, 1996, pages 45-48, XP000863239 cited in the application the whole document</p> <p>---</p>	1-24
Y	<p>A C TAS ET AL: "Characterization of virus infected cell cultures by pyrolysis / direct chemical ionization mass spectrometry" BIOMEDICAL AND ENVIRONMENTAL MASS SPECTROMETRY,GB,WILEY, LONDON, vol. 18, no. 9, page 757-760-760 XP002105697 ISSN: 0887-6134 the whole document</p> <p>---</p>	1-24
Y	<p>US 5 174 962 A (BRENNAN THOMAS M) 29 December 1992 (1992-12-29) the whole document</p> <p>---</p>	1-24
Y	<p>US 5 493 115 A (DEINZER MAX L ET AL) 20 February 1996 (1996-02-20) the whole document</p> <p>---</p>	1-24
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International Application No

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